

ANALYTICAL METHODS FOR MALACHITE GREEN

Completion Report
Malachite Green Analysis in Water

Prepared by

John L. Allen
Jane E. Gofus
Jeffery R. Meinertz

U.S. Fish and Wildlife Service
National Fisheries Research Center

Prepared for

Jerry Bauer, Project Manager
U.S. Department of Energy
Bonneville Power Administration
Division of Fish and Wildlife
P.O. Box 3621
Portland, OR 97208-3621

Project No. 89-040
Contract Number DE-A179-89BP96356

June 1991

Table of Contents

	<u>Page</u>
Abstract	2
Introduction	3
Description of the Project Area	3
Methods and Materials	4
Spectrophotometric Analysis	
High Performance Liquid Chromatography	4
Results and Discussion	7
Literature Search	7
Spectrophotometric Analysis	8
High Performance Liquid Chromatography	8
Sample Enrichment	8
Summary	14
Conclusions	16
Summary of Expenditures	17
 Appendix A	
Literature Review	18
 Appendix B	
¹⁴ C-Malachite Green Recovery from Water with Baker 10 Diol Columns	21
 Appendix C	
Standard curves and chromatograms of malachite green and leuco malachite green from well water samples spiked with 10 ppb, description of the calculation of ppb from ng found by HPLC, and the recovery of malachite green residues from well water samples spiked with 10 ppb each of malachite green and leuco malachite green . . .	36

	<u>Page</u>
Appendix D	
HPLC Minimum Detection Limit Raw Data for Chromatic and Leuco Malachite Green	42
Appendix E	
Chromatograms of Well Water Samples Spiked with the Carbinol and Leuco Forms of Malachite Green	44
Appendix F	
Recovery of Malachite Green Residues from Water of Various pHs . .	47
Appendix G	
Chromatogram of malachite green residues from pond water analyzed by HPLC with diol-column enrichment, recovery of malachite green residues from pond water samples spiked at 25.0, 50.0, and 100 ppb each of leuco malachite green and malachite green, and the water chemistry analysis of NFLX pond water	49
Tables	
Table 1. Mean percent recovery of ¹⁴C-malachite green and standard deviation (SD) from Baker 10 Diol columns after passage of 50-ml volumes of malachite green solutions (5, 10, 50, 100, or 500 ppb) through the column	9
Table 2. Mean percent recoveries and standard deviations for malachite green and leuco malachite green from well water spiked at 10 ppb each malachite green and leuco malachite green	10
Table 3. Mean percent recovery, mean concentration, standard deviation, variance, and minimum detection limit (MDL) for malachite green and leuco malachite green	10
Table 4. Mean recovery and standard deviation (STDS) of leuco malachite green (LEUCO) from well water spiked with 50 ppb of leuco malachite green	11
Table 5. Mean recovery and standard deviation (STDS) of malachite green carbinol from well water spiked with 50 ppb of carbinol	12
Table 6. Mean percent recoveries and standard deviations (STDS) of malachite green and leuco malachite green from waters of various pHs	13

	<u>Page</u>
Table 7. Mean percent recoveries and standard deviations (STDS) of malachite green residues from pond water spiked at concentrations of 25.0, 50.0, and 100 ppb each of leuco malachite green (LEUCO) and malachite green (MGR)	15
Table C-1. Example of the calculation to convert HPLC nanograms to parts per billion	40
Table C-2. Recovery of malachite green residues from well water samples spiked with 10 ppb each of malachite green (MGR) and leuco malachite green (LEUCO)	41
Table D.1. Raw data from the method detection limit (MDL) determination for the HPLC analysis of leuco malachite green (LEUCO) and malachite green (MGR) with diol-column enrichment according to the EPA procedure for the determination of the method detection limit.	43
Table F-1. Recoveries of malachite green residues from deionized water samples buffered to pH 6.0, 7.0, and 9.0 and well water samples with a pH of 8.0. All samples were spiked with 50.0 ppb of leuco malachite green (LEUCO) and 50.0 ppb of malachite green (MGR)	48
Table G-1. Recovery of malachite green residues from pond water spiked at concentrations of 25.0, 50.0, and 100 ppb each of leuco malachite green (LEUCO) and malachite green (MGR)	51
Table 6.2. Water chemistry analysis of NFLX pond water	52

Figures

Figure 1. Malachite green and the colorless forms which may be found in treated hatchery waters	3
Figure 2. Mean percent recoveries and standard deviations of leuco malachite green and malachite green from waters of various pH levels (Table 6)	13
Figure C-1. HPLC standard curves for leuco malachite green (A) and malachite green (B).	37
Figure C-2. HPLC analysis and chromatogram for a leuco malachite green and malachite green 10-ng standard made up in mobile phase.	38
Figure C-3. HPLC analysis and chromatogram of a 10-ppb sample extraB9	
Figure E-1. HPLC chromatogram and analysis of a well water sample spiked with 50-ppb malachite green carbinol	45

	<u>Page</u>
Figure E-2. HPLC chromatogram and analysis of a well water sample spiked with 50-ppb leuco malachite green	46
Figure G-1. HPLC chromatogram and analysis of pond water sample spiked with 100-ppb leuco malachite green and malachite green	50

Abstract

Malachite green is a known teratogen and therefore its use is limited to nonfood fish under an Investigational New Animal Drug permit (INAD), number 2573. Although a charcoal adsorption column was developed to remove malachite green from hatchery water, INAD compliance requires that the malachite green residue concentrations in any effluent from hatcheries using the chemical be quantified. Therefore, we developed a method for the analysis of malachite green residues in water.

Enrichment of the residues of malachite green in water on a diol column followed by High Performance Liquid Chromatographic (HPLC) analysis gives a minimum sensitivity of less than 10 ppb for the chemical. When combined with post-column oxidation using a lead oxide post-column reactor, the procedure can be used for the simultaneous analysis of malachite green in its leuco form, a decomposition product of the dye, as well as its chromatic form. Recovery of the leuco form is pH dependent and water samples should be adjusted to pH 6 to optimize recovery of this form. Water samples spiked with malachite green were concentrated on a diol column followed by elution with 0.05 M p-toluene sulfonic acid in methanol. The methanol elutes were analyzed by HPLC. Pond water samples spiked with malachite green and leuco malachite green yielded average recoveries of 95.4% for malachite green and 57.3% for leuco malachite green. Tap water samples spiked with the carbinol form of malachite green gave average recoveries of 98.6%. The method is very sensitive and is capable of detecting malachite green residues in water at less than 10 ppb.

Fish culturists, who cannot find an effective replacement for malachite green, can utilize the method to ensure that their effluents comply with INAD regulations.

Introduction

Malachite green is undoubtedly the most effective antifungal agent used in aquaculture. Due to potential health risks, the use of malachite green is currently limited to the treatment of nonfood fish under an Investigational New Animal Drug Application (INAD). Methods for determination of trace amounts of malachite green in hatchery water are needed to maintain the INAD. The methods of analysis must be capable of detecting malachite green and the breakdown products of the chemical. The two major breakdown products of malachite green are carbinol and leuco malachite green, both of which are colorless and therefore more difficult to detect. The carbinol form is an acid-base equilibrium product of malachite green in water. The leuco form is a reduction product of malachite green. The methods must also detect the parent chemical, malachite green, which is referred to as the chromatic or quinoidal form (Fig. 1).

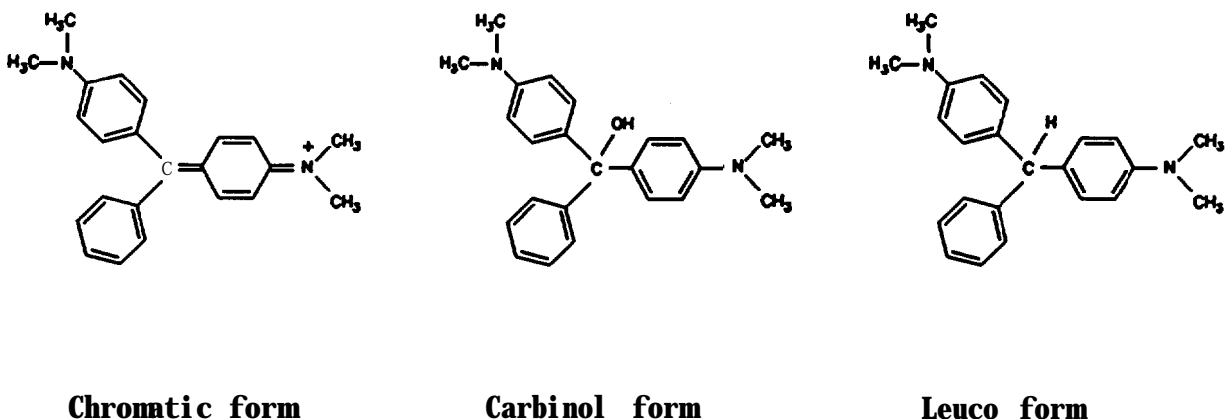


Figure 1. Malachite green and the colorless forms which may be found in treated hatchery waters.

Description of the Project Area

Control of fungal diseases on adult salmon and on incubating eggs is critical to the successful production of fingerling salmon. Malachite green is very effective for this. The U.S. Food and Drug Administration, however, requires data showing that only negligible amounts of malachite green are discharged from state and federal fish hatcheries. Under the INAD #2573, selected state and federal hatcheries are permitted to use malachite green if they can show little or no release in the hatchery effluent. The level must be low enough to satisfy regulatory concerns. The development of a method for the analysis of trace amounts of malachite green and its related products is necessary for maintaining the INAD.

Methods and Materials

Objective 1: Identify and test existing analytical methods for measuring malachite green analogs in water.

1

Task 1.1 Survey published literature for reported analytical methods.

A computerized search of the literature on analytical methods for triphenylmethane dyes was conducted using DIALOG. The literature on the use of malachite green in fisheries by Nelson (1974) was reviewed.

Task 1.2 Test each of the analytical approaches found in the literature, using known concentrations of each of the three malachite green analogs (vernal, carbinol, and leuco forms) to determine a statistically based recovery efficiency for each analytical approach.

Spectrophotometric Analysis

Spectrophotometric methods have been used for analysis of malachite green (vernal or chromatic form) because of the high absorbance of the chemical in the visible (618 nm) region of the spectrum

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) with a reverse-phase C_{18} column was investigated as a sensitive method to detect malachite green. Three mobile phases were evaluated. The first mobile phase consisted of various proportions of methanol and water. The second mobile phase consisted of various proportions of acetonitrile and water. Both mobile phases contained 0.05 M p-toluene sulfonic acid (TSA) as a counter ion. The third mobile phase consisted of an acetate buffer (about pH 4) in water mixed with different proportions of methanol.

Objective 2: Develop an original method that will detect all three forms of malachite green in distilled water with a detection limit of 10 ppb or less for each form and 10 ppb or less for "total malachite green."

Task 2.1 Evaluate High Performance Liquid Chromatographic methods for the detection of malachite green forms. Explore various methods of sample cleanup, extraction, and detectors.

HPLC with a reverse-phase C_{18} column was used for the detection of the malachite green residues. A mobile phase of 15% buffer (0.05 M sodium acetate and 0.1 M acetic acid in water) and 85% methanol at a flow rate of 2.0 mL/min was used for the HPLC solvent system. A lead oxide post-column oxidation reactor was used to oxidize leuco malachite green to the visual chromatic form. This system efficiently separates the malachite green and leuco malachite green peaks and both can be detected spectrophotometrically at 618 nm.

Recovery studies for malachite green were carried out in well water from the La Crosse National Fisheries Research Center (NFLX). Fifty-mL water samples spiked with 10.0 ppb each leuco malachite green and malachite green were aspirated through Baker 10 Diol extraction columns using low vacuum. The residues were eluted from the column with 2.0 mL of 0.05 M p-toluene sulfonic acid in methanol. The samples were analyzed by HPLC using a C_{18} column with a mobile phase of 15% buffer and 85% methanol at a flow rate of 2.0 mL/min. A reaction chamber containing 10% PbO_2 in Celite 545 was placed between the column and the spectrophotometric detector to oxidize the leuco form of malachite green to the chromatic form for detection at 618 nm. Standards of leuco malachite green and malachite green were prepared in mobile phase from methanol stock solutions of the chemicals.

Task 2.2 Determine limits of recovery and detection for each of the malachite forms and for "total malachite green."

Minimum limits of detection for malachite green and leuco malachite green in water were determined by the EPA method (U.S. Environmental Protection Agency, Federal Register, 49 [1984] 198). A 500-mL stock solution of 25.0 ppb each malachite green and leuco malachite green in well water was prepared. Seven 50.0-mL replicates from the sample stock were concentrated on the Baker 10 Diol column and analyzed using the HPLC method. The results of the analysis of the seven replicates were used to compute the minimum limits of detection for malachite green and leuco malachite green.

Task 2.3 If HPLC methods are not successful, repeat Tasks 2.1 and 2.2 using polarographic approaches.

HPLC appears to be the method of choice for the simultaneous analysis of the vernal (chromatic) and leuco form of malachite green.

Objective 3: Develop techniques to ensure the sensitivity and reliability of detection of 10 parts per billion or less in waters of a variety of water chemistries.

Task 3.1 Develop techniques to concentrate all three forms of malachite green from water and to extract them from the filter medium

Well water samples (pH 8.0) were spiked with 50.0 ppb of malachite green oxalate, leuco malachite green, and carbinol. The water samples were concentrated using the Baker 10 Diol columns and analyzed using the HPLC procedure developed in Task 2.1.

Task 3.2 Develop means to convert all three chemical forms to a common form so that the total malachite green present can be determined by the method(s) developed in Objective 2.

HPLC analysis detects the chromatic form of malachite green at 618 nm. Oxidation of leuco malachite green to the chromatic form for detection in the visible region of the spectrum was accomplished using a post-column reactor containing 10% (weight/weight) PbO_2 in Celite 545. The leuco malachite green is rapidly converted to the chromatic form in the post-column oxidation reactor and is detected at 618 nm with a spectrophotometric detector. The conversion of the carbinol form to the chromatic form is readily accomplished when 0.05 M p-toluene sulfonic acid in methanol comes into contact with the carbinol during elution from the diol column.

Task 3.3 Determine sensitivity and reliability of the analytical methods for malachite green in waters of pH 6.0, 7.0, and 8.0.

Deionized water samples were buffered to pH 6.0, 7.0, and 9.0 with 1.0 M NaOH and KH_2PO_4 . The well water samples were pH 8.0. The water samples at various pHs were spiked with 50.0 ppb of malachite green and leuco malachite green. Fifty-mL aliquots of the samples were concentrated on the Baker 10 Diol columns and the column eluates were analyzed using the HPLC procedure.

Objective 4: Determine if the developed method will accurately detect levels of malachite green at 10 ppb or less in actual hatchery effluents that contain feces, debris, biota, and metabolites.

Task 4.1 Develop protocols for collecting, preserving, and shipping water samples. Samples shall be collected from treated hatchery water ahead of the carbon filter and in the effluent from the filter.

Samples collected from a pond, tank, raceway, or hatchery effluent should be placed in brown glass bottles and stored in a refrigerator. Malachite green and its carbinol and leuco forms are susceptible to photolysis and should be protected from light. Malachite green is not being used on salmon hatcheries and no carbon filtration systems have been installed on salmon hatcheries, therefore no treated hatchery water was available for analysis.

Task 4.2 Determine sensitivity and reliability of the analytical method for malachite green in NFH water containing fish feces, debris, biota, clay turbidity, and metabolites.

Samples of water taken from a pond that contained fish were collected at the National Fisheries Research Center. The samples were spiked at 25.0, 50.0, and 100 ppb each of leuco malachite green and malachite green. Fifty-mL aliquots of the spiked samples were enriched on the Baker 10 Diol columns and the eluates from the columns were analyzed by the HPLC method.

Results and Discussion

Objective 1: Identify and test existing analytical methods for measuring malachite green analogs in water.

Task 1.1 Survey published literature for reported analytical methods.1

Literature Search

A literature search on methods for the analysis of malachite green and its major breakdown product, leuco malachite green, indicated the most applicable method of analysis for the compound was liquid chromatography with enrichment from water by column chromatographic concentration (see Appendix A).

Task 1.2 Test each of the analytical approaches found in the literature, using known concentrations of each of the three malachite green analogs (vernal, carbinol, and leuco forms) to determine a statistically based recovery efficiency for each approach.

Spectrophotometric Analysis

Spectrophotometric methods for the analysis of malachite green do not have the specificity needed for identification of the chemical, and leuco malachite green does not absorb light in the visible region of the spectrum. Further use of stand-alone spectrophotometric methods was abandoned.

High Performance Liquid Chromatography

HPLC with post-column oxidation and spectrophotometric detection (618 nm) was investigated for the analysis of malachite green and its analogs. The chromatic (vernal) and leuco forms of malachite green were easily separated into discrete peaks by HPLC with all the mobile phases evaluated. Mobile phases tested included acetonitrile and water and methanol and water. Both solutions contained 0.05 M TSA as a counter ion (Abidi 1983). Malachite green degradation was observed when TSA was used in the mobile phase in the presence of the post-column oxidation chamber. Subsequent use of the chemical in mobile phases was abandoned. A solvent system of sodium acetate and acetic acid buffer in water and methanol was also investigated and proved to be superior for analysis. The system used by Roybal et al. (1989) for separation of gentian violet and its leuco form also yielded baseline separation of malachite green and its leuco form.

Sample Enrichment

The Baker 10 extraction columns were evaluated visually for retention of malachite green. Columns with diol solid phase retained malachite green in a narrow band. The entire band was eluted from the column with 2.0 mL of 0.05 M TSA in methanol. Unlike mobile phases in which TSA was used, no decomposition of malachite green was observed in developing chromatograms when 0.05 M TSA was used only for elution of the diol columns. The Baker 10 Diol column was tested for extraction efficiency using ^{14}C -malachite green solutions adjusted to approximately 5,000 dpm. Concentrations ranging from 5 to 500 ppb were tested for sample extraction and recovery efficiency. Mean recoveries of malachite green ranged from 88.4% at 10 ppb to 107.6% at 5 ppb (Table 1). Approximately 3.5% of the malachite green in all solutions tested was not retained on the column, but washed through the column and was recovered in the effluent water (Table 1, Appendix B).

Table 1. Mean percent recovery of 14C-malachite green and standard deviation (SD) from Baker 10 Diol columns after passage of 50-mL volumes of malachite green solutions (5, 10, 50, 100, or 500 ppb) through the column.

Concentration malachite green (ppb)	Percent activity not retained during elution	Percent activity recovered from methanol-TSA eluate
5	3.7 (0.13)	107.6 (5.48)
10	3.2 (0.13)	88.4 (11.1)
50	3.2 (0.14)	98.6 (1.98)
100	3.4 (0.13)	101.2 (2.51)
500	3.6 (0.10)	97.6 (2.74)

Objective 2: Develop an original method that will detect all three forms of malachite green in distilled water with a detection limit of 10 ppb or less for each form and 10 ppb or less for "total malachite green."

Task 2.1 Evaluate High Performance Liquid Chromatographic methods for the detection of malachite green forms. Explore various methods of sample cleanup, extraction, and detectors.

The 10-ppb recovery study used well water spiked with both leuco malachite green and malachite green. Seven replicate samples gave a mean total recovery of 77.3% for malachite green plus leuco malachite green with a standard deviation of 5.21. The mean recovery of leuco malachite green was 45.0% with a standard deviation of 4.25. The mean recovery of malachite green was 110% with a standard deviation of 7.59 (Table 2). The individual recoveries can be seen in Appendix C.

Included in Appendix C are standard curves, representative chromatograms of both a sample and a standard, formulas for the calculation of ppb from ng detected by HPLC, and the individual recoveries of malachite green residues from water samples spiked with 10 ppb each of malachite green and leuco malachite green.

Over 600 injections have been made on the HPLC system with the PbO₂ post-column reaction chamber without an apparent change in its ability to convert leuco malachite green to the chromatic form

Table 2. Mean percent recoveries and standard deviations for malachite green and leuco malachite green from well water spiked at 10 ppb each of malachite green and leuco malachite green.

Chemical form	Mean percent recovered	Standard deviation
LEUCO	45.0	4.25
MGR	110	7.59
LEUCO + MGR	77.3	5.21

Task 2.2 Determine limits of recovery and detection for each of the malachite forms and for "total malachite green."

The minimum detection limit was determined using seven replicate samples of well water spiked with 25.0 ppb each of leuco malachite green and malachite green (Appendix D).

The mean percent recovery of leuco malachite green was 48.2% with a standard deviation of 2.58% and a minimum detection limit of 2.01 ppb. The mean recovery of malachite green was 78.3% with a standard deviation of 3.62% and a minimum detection limit of 2.83 ppb (Table 3).

Table 3. Mean percent recovery, mean concentration, standard deviation, variance, and minimum detection limit (MDL) for malachite green and leuco malachite green.

Chemical form	Mean % recovered (stds)	Mean conc (ppb)	Standard deviation (ppb)	Variance (ppb)	Detection* limits (ppb)
LEUCO	48.2 (2.58)	12.1	0.64	0.42	2.01
MGR	78.3 (3.62)	19.6	0.90	0.82	2.83

^aMDL = (3.143) X (standard deviation).

Task 2.3 If HPLC methods are not successful, repeat Tasks 2.1 and 2.2 using polarographic approaches.

HPLC appears to be the method of choice for the simultaneous analysis of the vernal (chromatic) and leuco form of malachite green.

Objective 3: Develop techniques to ensure the sensitivity and reliability of detection of 10 parts per billion or less in waters of a variety of water chemistries.

Task 3.1 Develop techniques to concentrate all three forms of malachite green from water and to extract them from the filter medium

Well water samples spiked with 50.0 ppb leuco malachite green were analyzed in triplicate. The mean recovery for the three samples was 55.7% with a standard deviation of 2.64 (Table 4). Leuco malachite green recoveries were low. The lower recovery of leuco malachite green is possibly due to photolysis or other types of decomposition, and possible adsorption to glass and plastic surfaces.

Table 4. Mean recovery and standard deviation (STDS) of leuco malachite green (LEUCO) from well water spiked with 50 ppb of leuco malachite green.

Sample number	Leuco		
	Conc. recovered (ppb)	Percent recovered	Mean % recovered (stds)
1	26.4	52.7	55.7 (2.64)
2	28.7	57.5	
3	28.5	57.0	

Well water samples spiked with 50.0 ppb carbinol were also analyzed in triplicate (Appendix E). The mean recovery for the carbinol samples was 98.6% with a standard deviation of 2.18 (Table 5).

Table 5. Mean recovery and standard deviation (STDS) of malachite green carbinol from well water spiked with 50 ppb of carbinol.

Sample number	MGR	Carbinol		
	Conc. recovered (ppb)	Conc. ^a recovered (ppb)	Percent recovered	Mean recovery (stds)
1	57.2	49.1	98.2	98.6 (2.18)
2	59.1	50.7	101	
3	56.3	48.4	96.7	

$$\text{MGR to Carbinol Conversion Factor} = \frac{\text{Mbl. Wt. of Carbinol (346.48)}}{\text{Mbl. Wt. of MGR Oxalate (927.02)/2}} = 0.85866$$

Carbinol is detected as malachite green because it is converted to the chromatic form during the diol extraction procedure with p-toluene sulfonic acid and methanol (Task 3.1). The leuco form is converted to the chromatic form during the post-column oxidation as shown in Task 3.1.

Task 3.2 Develop means to convert all three chemical forms to a common form so that the total malachite green present can be determined by the method(s) developed in objective 2.

Both leuco malachite green and carbinol were converted to the chromatic form of malachite green to enable HPLC detection at 618 nm Sample chromatograms are shown in Appendix E.

Task 3.3 Determine sensitivity and reliability of the analytical methods for malachite green in waters of pH 6.0, 7.0, and 8.0.

Deionized water samples were buffered to pH 6.0, 7.0, and 9.0 with 1.0 M NaOH and KH_2PO_4 . The well water samples were pH 8.0. The water samples at various pHs were spiked with 50.0 ppb of malachite green and leuco malachite green and analyzed using the HPLC procedure with diol-column extraction.

Three samples of the deionized water, buffered to pH 6.0, 7.0, or 9.0 were analyzed for malachite green and leuco malachite green. Six samples of

pH 8.0 well water were also analyzed for malachite green and leuco malachite green (Appendix F). The mean recoveries for leuco malachite green at pH 6.0, 7.0, 8.0, and 9.0 were 61.2%, 50.1%, 48.3%, and 39.1%, respectively. The mean recoveries for malachite green at pH 6.0, 7.0, 8.0, and 9.0 were 98.3%, 91.7%, 98.3%, and 99.3%, respectively (Table 6). Malachite green recovery did not appear to be affected by pH in this study. However, the recovery of leuco malachite green ranged from 39.1% at pH 9 to 61.2% at pH 6 (Fig. 2). The pH of water samples should be adjusted to pH 6 before extraction in order to obtain the optimum recovery of leuco malachite green.

Table 6. Mean percent recoveries and standard deviations (STDS) of malachite green and leuco malachite green from waters of various pHs.

Chemical form	Mean percent recovery			
	pH 6 (stds)	pH 7 (stds)	pH 8 (stds)	pH 9 (stds)
LEUCO	61.2 (2.89)	50.1 (2.72)	48.3 (7.86)	39.1 (0.90)
MGR	98.3 (2.87)	91.7 (5.64)	98.3 (5.59)	99.3 (1.98)
LEUCO t MGR	79.7 (2.10)	70.9 (4.06)	73.3 (6.51)	69.2 (0.54)

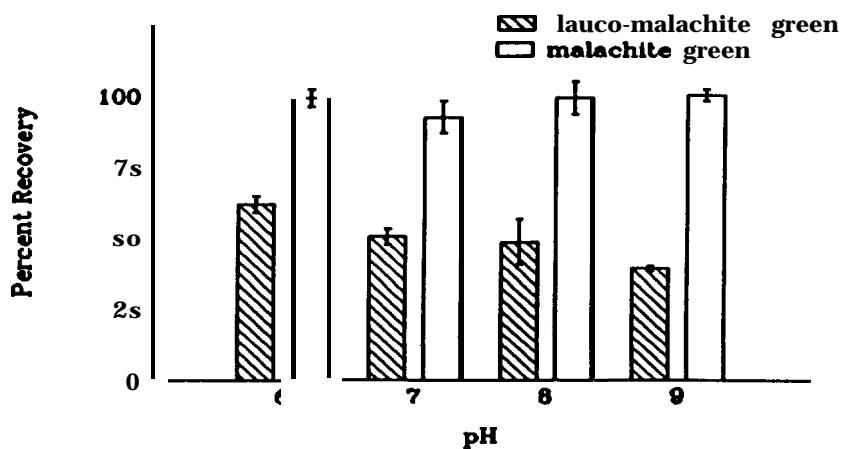


Figure 2. Mean percent recoveries and standard deviations of leuco malachite green and malachite green from waters of various pH levels (Table 6).

Objective 4: Determine if the developed method will accurately detect levels of malachite green at 10 ppb or less in actual hatchery effluents that contain feces, debris, biota, and metabolites.

Task 4.1 Develop protocols for collecting, preserving, and shipping water samples. Samples shall be collected from treated hatchery water ahead of the carbon filter and in the effluent from the filter.

Samples collected from a pond, tank, raceway, or hatchery effluent should be placed in brown glass bottles and stored in a refrigerator. Malachite green and its carbinol and leuco forms are susceptible to photolysis and should be protected from light. Malachite green is not being used on salmon hatcheries and no carbon filtration systems have been installed on salmon hatcheries. Therefore, no samples were collected and analyzed from hatchery water. The pond water spiked with malachite green simulates actual hatchery water.

Task 4.2 Determine sensitivity and reliability of the analytical method for malachite green in NFH water containing fish feces, debris, biota, clay turbidity, and metabolites.

Pond water spiked at 25.0, 50.0, and 100 ppb each of leuco malachite green and malachite green were analyzed using the HPLC method after sample enrichment on Baker 10 Diol columns. The total recoveries of malachite green plus leuco malachite green were 77.4%, 74.0%, and 79.8% at spiking concentrations of 25.0, 50.0, and 100 ppb, respectively. Mean recoveries of leuco malachite green at 25.0, 50.0, and 100 ppb were 58.5%, 50.8%, and 62.5%, respectively. Mean recoveries of malachite green at 25.0, 50.0, and 100 ppb were 96.23, 93.0%, and 97.0%, respectively (Table 7). The concentration of the spike had little influence on the recovery of the malachite green residues in pond water. No interferences in the analysis were noted from the pond water samples. Chromatograms of pond water samples are shown in Appendix G.

Summary

Well water spiked with 10.0 ppb each of malachite green and leuco malachite green followed by HPLC analysis with sample enrichment using the Baker 10 Diol column gave an average extraction and recovery of 110% and 45.0%, respectively. The total recovery of malachite green plus leuco malachite green averaged 77.3%. The lower recovery of leuco malachite green is possibly due to photolysis or other types of decomposition, and possible adsorption to glass and plastic surfaces. Well water spiked with 50.0 ppb of leuco malachite green gave an average recovery 55.7%.

Table 7. Mean percent recoveries and standard deviations (STDS) of malachite green residues from pond water spiked at concentrations of 25.0, 50.0, and 100 ppb each of leuco malachite green (LEUCO) and malachite green (MGR).

Chemical form	Mean percent recovered at:		
	25 ppb ^a	50 ppb (stds)	100 ppb (stds)
LEUCO	58.5	50.8 (4.67)	62.5 (0.89)
MGR	96.2	93.0 (3.52)	97.0 (2.30)
LEUCO t MGR	77.4	74.0 (3.78)	79.8 (0.85)

^a Only two samples were analyzed at the 25-ppb level and no standard deviation was calculated.

The acidic conditions of the extraction and analysis convert any carbinol form of malachite green to the chromatic form and it is then detected as malachite green. Water samples spiked with 50.0 ppb of malachite green carbinol gave an average recovery of 98.6%.

The method minimum detection concentration was determined by analyzing seven replicate samples of water spiked with 25.0 ppb each of malachite green and leuco malachite green. The minimum detection level was 2.83 ppb for malachite green and 2.01 ppb for leuco malachite green.

The effect of pH on the recovery of malachite green and leuco malachite green was investigated by analyzing samples of water buffered to various pHs. Three samples of the deionized water buffered to pH 6.0, 7.0, and 9.0 were spiked with 50.0 ppb of leuco malachite green and malachite green. Six samples of pH 8.0 well water were also spiked with 50.0 ppb of malachite green and leuco malachite green. The samples were analyzed for residues of malachite green. The mean recovery of leuco malachite green at pH 6.0, 7.0, 8.0, and 9.0 were 61.2%, 50.1%, 48.3%, and 39.1%, respectively. The mean recovery of malachite green at pH 6.0, 7.0, 8.0, and 9.0 were 98.33, 91.7%, 98.3%, and 99.3%, respectively. This indicates that the method is most effective on water samples of pH 6. Malachite green recovery did not appear to be affected by pH in this study. However, the recovery of leuco malachite green ranged from 39.1% at pH 9 to 61.2% at pH 6. The pH of water samples should be adjusted to pH 6 before extraction in order to obtain the optimum recovery of leuco malachite green.

Water from a pond which contained fish at the National Fisheries Research Center was spiked with 25.0, 50.0, and 100 ppb each of malachite green and leuco malachite green. Mean recoveries of leuco malachite green at 25.0, 50.0, and 100 ppb were 58.5%, 50.8%, and 62.5%, respectively. Mean recoveries of malachite green at 25.0, 50.0, and 100 ppb were 96.23, 93.0%, and 97.0%, respectively. The total recoveries of malachite green plus leuco malachite green were 77.43, 74.0%, and 79.8% at spiking concentrations of 25.0, 50.0, and 100 ppb, respectively. The concentration of the spike had little influence on the recovery of the malachite green residues in pond water. No interferences in the analysis were noted from the pond water samples.

The HPLC method of analysis with residue enrichment on a Baker 10 Diol column is capable of detecting residues of malachite green at concentrations of less than 10 ppb. The method requires residue enrichment followed by HPLC for separation of the leuco and chromatic forms and detection of the residues with a spectrophotometric detector at 618 nm. The detection at 618 nm gives the advantage of high sensitivity for this dye and increased specificity for the chemical in this region of the spectrum.

A single HPLC pump, injector, C_{18} column, post-column oxidizer, variable wavelength detector, and a recorder or data system make up the instrumentation needed for the analysis. The fabrication of the post-column oxidation reactor requires no special equipment.

Conclusions

The HPLC equipment needed for the method includes as follows: a single HPLC pump, injector, C_{18} column, post-column oxidizer, variable wavelength detector, and a recorder or data system. The fabrication of the post-column oxidation reactor, which is not commercially available, requires no special equipment.

The method is simple and rapid. Extraction of the residues of malachite green from water on a Baker 10 Diol column and subsequent elution of the residues from the column in a small volume of 0.05 M p-toluene sulfonic acid takes approximately 20 minutes. HPLC analysis, after standards have been run, takes about 17 minutes per sample.

The method shows good reproducibility and will detect residues of malachite green present in water in the form of the parent chemical and in its leuco or carbinol forms. The method is capable of determining residues of malachite green at concentrations of less than 10 ppb in water. The recovery of leuco malachite green is low but the reproducibility and high sensitivity of the method for this form of the chemical allow its detection at very low concentrations.

Summary of Expenditures
Billed to BPA as of March 21, 1991

Denver Finance Center Overhead	\$ 24,099.98
Equipment, HPLC	34,863.23
Services and Supplies	17,319.00
Travel	723.00
National Fisheries Research Center Overhead	2,247.87
Salaries	<u>81,407.67</u>
Cumulative Invoiced Expenditures to Date	\$160,660.00

Appendix A

Analytical Methods for Malachite Green

Project 89-040

Literature Review

References

- Abidi S. L. 1983. High-performance liquid chromatography of quinoidal iminium compounds derived from triphenylmethanes. *Journal of Chromatographic Science* 225:101-114.
- Clifton-Hadley, R. S., and D. L. Alderman. 1987. The effect of malachite green upon proliferative kidney disease. *Journal of Fish Diseases* 10:101-107.
- Alderman, D. J. 1985. Malachite green: a review. *Journal of Fish Diseases* 8:289-298.
- Bauer K., H. Dandgschat, H. O. Knoeppler, and J. Neudegger. 1988. Intake and elimination of malachite green in rainbow trout. *Lebensmittelhygiene* 39:97-102.
- Dawson, V. K. 1982. A rapid high-performance liquid-chromatographic method for simultaneously determining the concentration of TFM and Baker 73 in water during lampricide treatments. *Canadian Journal of the Fisheries and Aquatic Science* 39:779-782.
- Dawson, V. K., P. D. Harman, D. P. Schultz, and J. L. Allen. 1983. A rapid method for measuring rotenone in water a piscicidal concentrations. *Transactions of the American Fisheries Society* 112:725-727.
- Martinez, E. E., and W Shimoda. 1989. Mdified liquid chromatographic method for determination of gentian violet in animal feed. *Journal of the Association of Official Analytical Chemists* 72:742-745.
- Minns, R. K., J. E. Roybal, J. A. Hurabut, and W Shimoda. 1990. Rapid method for determination of leucogentian violet in chicken fat by liquid

- chromatography with electrochemical detection. **Journal of the Association of Official Analytical Chemists** 46:705-708.
- Nelson, C. R., and R. A. Hites. 1980. Aromatic amines in and near the Buffalo River. **Environmental Science and Technology** 14:1147-1149.
- Nelson, N. C. 1974. A review of the literature on the use of malachite green in fisheries. National Technical Information Service, Springfield Virginia. PB 235-450/AS. 79 pp.
- Roybal, J. E., R. K. Munns, J. A. Hurlbut, and W. Shinoda. 1989. High-performance liquid chromatography of gentian violet, its demethylated metabolites, leucogentian violet and methylene blue with electrochemical detection. **Journal of Chromatography** 467:259-266.
- Roybal, J. E., R. K. Munns, J. A. Hurlbut, and W. Shinoda. 1990. Determination of gentian violet, its demethylated metabolites, and leucogentian violet in chicken tissue by liquid chromatography with electrochemical detection. **Journal of the Association of Official Analytical Chemists** 73:940-946.
- Rushing, L. G., and M. C. Bowman. 1980. Determination of gentian violet in animal feed human urine, and wastewater by high pressure liquid chromatography. **Journal of Chromatographic Science** 18:224-232.

Appendix B
Analytical Methods for Malachite Green
Project 89-040

**¹⁴C-Malachite Green Recovery from
Water with Baker 10 Diol Columns**

14C-Malachite Green Extraction From Water - STANDARD SOLUTION SAMPLES

USER#: 0 6 DATE: FEB 02 1989 Study N&r..... MCR8901
 ID: MCR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File information: 14C-Malachite Green Extraction From Water--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM/2ml - BKG	MEAN DPM/2ml (SD)	DPM/50ml
0.5ppm Std Soln 1	2061.99	2010.92		
0.5ppm Std Soln 2	2071.64	2020.56		
0.5ppm Std Soln 3	2014.42	1963.34		
0.5ppm Std Soln 4	2068.98	2017.90		
0.5ppm Std Soln 5	2073.03	2021.95	2006.93 24.74	50173.33
0.1ppm Std Soln 1	1851.15	1800.07		
0.1ppm Std Soln 2	1977.29	1926.21		
0.1ppm Std Soln 3	1917.43	1866.35		
0.1ppm Std Soln 4	1985.00	1933.92		
0.1ppm Std Soln 5	1992.50	1941.43	1893.60 60.14	47339.93
0.05ppm Std Soln 1	1937.69	1886.61		
0.05ppm Std Soln 2	1974.36	1923.28		
0.05ppm Std Soln 3	1992.33	1941.25		
0.05ppm Std Soln 4	2040.50	1989.42		
0.05ppm Std Soln 5	2032.26	1981.19	1944.35 42.36	48608.75

14C-Malachite Green Extraction From Water - 0.5 ppm WASH SAMPLES

USER#: 0 6 DATE: FEB 02 1989 Study N & r..... MGR8901
 ID: HGR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File infommition: 14C-Malachite Green Extraction From Water--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM - BKG	TOTAL DPM IN WASH	% OF APPLIED DPM	AVG % OF APPLIED DPM (SD)
Run#1 0.5ppm Uash1	377.69	327.33			
Run#1 0.5ppm Wash2	383.38	333.02			
Run#1 0.5ppm Uash3	394.16	343.80			
Run#1 0.5ppm Wash4	391.43	341.08			
Run#1 0.5ppm Wash5	413.33	362.98	1708.22	3.40	
Run#2 0.5ppm Uash1	418.92	368.56			
Run#2 0.5ppm Wash2	403.15	352.79			
Run#2 0.5ppm Uash3	419.50	369.14			
Run#2 0.5ppm Uash4	417.94	367.59			
Run#2 0.5ppm Wash5	398.20	347.84	1805.93	3 . 6 8	
Run#3 0.5ppm Uash1	433.25	382.89			
Run#3 0.5ppm Wash2	425.10	374.75			
Run#3 0.5ppm Wash3	417.09	366.73			
Run#3 0.5ppm Uash4	425.76	375.41			
Run#3 0.5ppm Wash5	367.51	317.15	1816.94	3.62	
Run#4 0.5ppm Uash1	422.86	372.51			
Run#4 0.5ppm Wash2	406.86	356.51			
Run#4 0.5ppm Uash3	418.62	368.26			
Run#4 0.5ppm Wash4	406.83	356.48			
Run#4 0.5ppm Wash5	370.50	320.14	1773.90	3.54	
Run#5 0.5ppm Uash1	418.02	367.66			
Run#5 0.5ppm Wash2	418.81	368.46			
Run#5 0.5ppm Uash3	417.56	367.20			
Run#5 0.5ppm Wash4	419.67	369.32			
Run#5 0.5ppm Uash5	384.36	334.01	1806.65	3.60	
					3.55
					0.09

14C-Malachite Green Extraction From Uater - 0.5 ppm FLUSH SAMPLES

USER#: 0 6 DATE: FEB 02 1989 Study Number..... MGR8901
 ID: MGR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File information: 14C-Malachite Green Extraction From Water--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM/50ul - BKG	MEAN DPM/50ul - BKG (SD)	TOTAL DPM IN FLUSH	% OF APPLIED DPM	AVG % OF APPLIED DPM (SD)
Run#1 0.5ppm Flush1	1238.93	1157.48				
Run#1 0.5ppm Flush2	1244.46	1163.01				
Run#1 0.5ppm Flush3	1279.10	1197.65	1172.72 21.77	46908.65	93.49	
Run#2 0.5ppm Flush1	1341.20	1259.75				
Run#2 0.5ppm Flush2	1348.30	1266.85				
Run#2 0.5ppm Flush3	1345.34	1263.89	1263.49 3.56	50539.78	100.73	
Run#3 0.5ppm Flush1	1329.56	1248.11				
Run#3 0.5ppm Flush2	1304.84	1223.39				
Run#3 0.5ppm Flush3	1288.86	1207.41	1226.30 20.50	49052.15	97.77	
Run#4 0.5ppm Flush1	1280.63	1199.18				
Run#4 0.5ppm Flush2	1271.10	1189.65				
Run#4 0.5ppm Flush3	1328.22	1246.78	1211.87 30.60	48474.86	96.61	
Run#5 0.5ppm Flush1	1282.36	1200.91				
Run#5 0.5ppm Flush2	1315.34	1233.89				
Run#5 0.5ppm Flush3	1378.54	1297.09	1243.96 48.88	49758.49	99.17	
						97.56 2.74

14C-Malachite Green Extraction From Uater - 0.05 ppm WASH SAMPLES

USER#: 06 DATE: FEB 02 1989 Study Number..... MGR8901
 ID: MGR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File infomtion: 14C-Malachite Green Extraction From Uater--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM - BKG	TOTAL DPM IN WASH	% OF APPLIED DPM	AVG % OF APPLIED DPM (SD)
Run#1 0.05ppm Uash1	383.84	333.48			
Run#1 0.05ppm Wash2	384.50	334.14			
Run#1 0.05ppm Uash3	405.16	354.81			
Run#1 0.05ppm Uash4	382.33	331.97			
Run#1 0.05ppm Wash5	326.34	275.98	1630.39	3.35	
Run#2 0.05ppm Uash1	407.64	357.29			
Run#2 0.05ppm Wash2	400.84	350.48			
Run#2 0.05ppm Wash3	408.06	357.71			
Run#2 0.05ppm Uash4	422.76	372.41			
Run#2 0.05ppm Wash5	356.31	305.95	1743.84	3.59	
Run#3 0.05ppm Uash1	404.28	353.93			
Run#3 0.05ppm Wash2	411.87	361.52			
Run#3 0.05ppm Uash3	404.76	354.41			
Run#3 0.05ppm Wash4	403.10	352.74			
Run#3 0.05ppm Wash5	395.70	345.35	1767.95	3.64	
Run#4 0.05ppm Uash1	417.58	367.23			
Run#4 0.05ppm Wash2	419.54	369.19			
Run#4 0.05ppm Uash3	402.88	352.53			
Run#4 0.05ppm Uash4	452.18	401.83			
Run#4 0.05ppm Wash5	358.41	308.06	1798.83	3.70	
Run#5 0.05ppm Wash1	455.92	405.57			
Run#5 0.05ppm Wash2	462.33	411.98			
Run#5 0.05ppm Wash3	452.13	401.77			
Run#5 0.05ppm Wash4	442.23	391.87			
Run#5 0.05ppm Wash5	425.12	374.77	1985.96	4.09	

3.67
0.27

14C-Malachite Green Extraction From Water - 0.05 ppm FLUSH SAMPLES

USER#: 0 6 DATE: FEB 02 1989 Study Nuder..... MGR8901
 ID: MGR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File information: 14C-Malachite Green Extraction From Water--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM/50ul - BKG	MEAN DPM/50ul - BKG (SD)	TOTAL DPM IN FLUSH	% OF APPLIED DPM	AVG % OF APPLIED DPH (SD)
Run#1 0.05ppm Flush1	1218.60	1137.15				
Run#1 0.05ppm Flush2	1222.71	1141.26				
Run#1 0.05ppm Flush3	1204.25	1122.80	1133.74 9.69	45349.53	93.29	
Run#2 0.05ppm Flush1	1302.82	1221.37				
Run#2 0.05ppm Flush2	1310.18	1228.73				
Run#2 0.05ppm Flush3	1278.65	1197.20	1215.77 16.50	48630.81	100.05	
Run#3 0.05ppm Flush1	1244.46	1163.01				
Run#3 0.05ppm Flush2	1229.48	1148.03				
Run#3 0.05ppm Flush3	1228.99	1147.54	1152.86 8.79	46114.51	94.87	
Run#4 0.05ppm Flush1	1183.65	1102.20				
Run#4 0.05ppm Flush2	1285.56	1204.11				
Run#4 0.05ppm Flush3	1190.58	1109.13	1138.48 56.94	45539.22	93.69	
Run#5 0.05ppm Flush1	1106.64	1025.19				
Run#5 0.05ppm Flush2	1195.68	1114.23				
Run#5 0.05ppm Flush3	1048.41	966.96	1035.46 74.17	41418.54	85.21	
						93.42
						5.33

14C-Malachite Green Extraction From Uater - 0.1 ppm WASH SAMPLES

USER#: 0 6 **DATE:** FEB 02 1989 **Study Nuder.....** MGR8901
IO: MGR-TLC **TIME:** 15:06 **Proofed by.....** JM 2/21/89
BSF version: 2 **DATE:** FEB 06 1989 **Lab notebook # and pages...** Pages 5-11
Instrument type: LS5801 **TIME:** 11:16
Filename: A:\14C-MGR\WATEXGP1.wk1
File information: 14C-Malachite Green Extraction From Water--Group 1
Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM - BKG	TOTAL OPM IN WASH	% OF APPLIED DPM	AVG % OF APPLIED DPM (SD)
Run#1 0.1ppm Uash1	364.79	314.44			
Run#1 0.1ppm Wash2	375.70	325.35			
Run#1 0.1ppm Uash3	371.02	320.67			
Run#1 0.1ppm Wash4	382.03	331.68			
Run#1 0.1ppm Wash5	332.30	281.95	1574.09	3.33	
Run#2 0.1ppm Uash1	391.27	340.91			
Run#2 0.1ppm Wash2	385.43	335.07			
Run#2 0.1ppm Uash3	378.19	327.84			
Run#2 0.1ppm Wash4	391.13	340.78			
Run#2 0.1ppm Uash5	325.67	275.32	1619.93	3.42	
Run#3 0.1ppm Wash1	354.40	304.05			
Run#3 0.1ppm Wash2	361.08	310.72			
Run#3 0.1ppm Uash3	358.56	308.21			
Run#3 0.1ppm Wash4	370.03	319.68			
Run#3 0.1ppm Wash5	334.52	284.17	1526.83	3.23	
Run#4 0.1ppm Uash1	384.83	334.47			
Run#4 0.1ppm Wash2	378.13	327.78			
Run#4 0.1ppm Uash3	399.85	349.49			
Run#4 0.1ppm Wash4	395.46	345.10			
Run#4 0.1ppm Wash5	338.41	288.06	1644.91	3.47	
Run#5 0.1ppm Uash1	392.75	342.40			
Run#5 0.1ppm Wash2	394.04	343.69			
Run#5 0.1ppm Wash3	396.41	346.06			
Run#5 0.1ppm Uash4	404.08	353.73			
Run#5 0.1ppm Wash5	348.16	297.81	1683.68	3.56	
					3.40
					0.13

14C-Malachite Green Extraction From Uater - 0.1 ppm FLUSH SAMPLES

USER#: 0 6 DATE: FEB 02 1989 Study Number..... MGR8901
 ID: MGR-TLC TIME: 15:06 Proofed by..... JM 2/21/89
 BSF version: 2 DATE: FEB 06 1989 Lab notebook # and pages... Pages 5-11
 Instrument type: LS5801 TIME: 11:16
 Filename: A:\14C-MGR\WATEXGP1.wk1
 File information: 14C-Malachite Green Extraction From Uater--Group 1
 Extracted from A:\UN06-001.BSF

SAMPLE ID	DPM CHANNEL 1	DPM/50ul - BKG	MEAN DPM/50ul - BKG (SD)	TOTAL DPM IN FLUSH	% OF APPLIED DPM	AVG % OF APPLIED DPM (SD)
Run#1 0.1ppm Flush1	1219.82	1138.37				
Run#1 0.1ppm Flush2	1236.09	1154.64				
Run#1 0.1ppm Flush3	1234.95	1153.50	1148.84 9.08	45953.46	97.07	
Run#2 0.1ppm Flush1	1303.02	1221.57				
Run#2 0.1ppm Flush2	1280.76	1199.31				
Run#2 0.1ppm Flush3	1283.70	1202.25	1207.71 12.09	48308.50	102.05	
Run#3 0.1ppm Flush1	1307.32	1225.87				
Run#3 0.1ppm Flush2	1314.23	1232.78				
Run#3 0.1ppm Flush3	1305.49	1224.05	1227.57 4.61	49102.65	103.72	
Run#4 0.1ppm Flush1	1278.29	1196.84				
Run#4 0.1ppm Flush2	1290.15	1208.70				
Run#4 0.1ppm Flush3	1302.37	1220.92	1208.82 12.04	48352.83	102.14	
Run#5 0.1ppm Flush1	1229.05	1147.60				
Run#5 0.1ppm Flush2	1309.90	1228.45				
Run#5 0.1ppm Flush3	1287.22	1205.77	1193.94 41.70	47757.67	100.88	
						101.17 2.51

14C Malachite Green Extraction From Water - GROUP 2 EXTRACTION-- ANALYSIS OF STANDARD SOLUTIONS

USER#: 06 DATE: FEB 17 1989 Study Number.....MGR8901
 ID: MGR-TLC TIME: 07:27 Proofed by..... JM 3/8/89
 BSF version: 2 DATE: FEB 22 1989 Lab notebook # and pages... Pages 14-16
 Instrument type: LS5801 TIME: 08:24
 Filename: A:\14C-MGR\WATEXGP2.WK1
 File information: 14C-Malachite Green Extraction From Water--Group 2
 Extracted from A:\UN06-003.BSF

SAMPLE NUMBER	POSITION NUMBER	SAMPLE ID	DPM CHANNEL 1 DPM/2ml - BKG	MEAN DPM/2ml (SD)	DPM/50ml
55 44- 1		0.05ppm Std Soln 1	4019.47	3965.71	
56 44- 2		0.05ppm Std Soln 2	4215.29	4161.53	
57 44- 3		0.05ppm Std Soln 3	4267.26	4213.51	
58 44- 4		0.05ppm Std Soln 4	4331.74	4277.99	
59 44- 5		0.05ppm Std Soln 5	4339.85	4286.09	4180.97 104524.17 130.61
60 44- 6		0.01ppm Std Soln 1	900.90	847.15	
61 44- 7		0.01ppm Std Soln 2	905.35	851.60	
62 44- 8		0.01ppm Std Soln 3	907.63	853.88	
63 44- 9		0.01ppm Std Soln 4	910.14	856.39	
64 44-10		0.01ppm Std Soln 5	901.80	848.04	851.41 21285.28 3.89
65 44-11		0.005ppm Std Soln 1	397.86	344.10	
66 44-12		0.005ppm Std Soln 2	418.96	365.21	
67 60- 1		0.005ppm Std Soln 3	418.64	364.88	
68 60- 2		0.005ppm Std Soln 4	428.67	374.91	
69 60- 3		0.005ppm Std Soln 5	424.41	370.66	363.95 9098.81 11.85

USER#: 06	DATE: FEB 17 1989	Study Number.....	MGR8901
IO: MGR-TLC	TIME: 07:27	Proofed by.....	JM 3/8/89
BSF version: 2	DATE: FEB 22 1989	Lab notebook # and pages...	Pages 14-16
Instrument type: LS5801	TIME: 08:24		
Filename: A:\14C-MGR\WATEXGP2.WK1			
File information: 14C-Malachite Green Extraction From Water--Group 2			
Extracted from A:\UN06-003.BSF			

30

14C Malachite Green Extraction From Water - GROUP 2 EXTRACTION-- ANALYSIS OF 0.05 ppm FLUSH SAMPLES

USER#: 0 6 DATE: FEB 17 1989 Study Number..... MGR8901
 ID: HGR-TLC TIME: 07:27 Proofed by..... JM 3/8/89
 BSF version: 2 DATE: FEB 22 1989 Lab notebook # and pages... Pages 14-16
 Instrument type: LS5801 TIME: 08:24
 Filename: A:\14C-MGR\WATEXGP2.WK1
 File information: 14C-Malachite Green Extraction From Water--Group 2
 Extracted from A:\UN06-003.BSF

SAMPLE POSITION NUMBER	NUMBER	SAMPLE ID	DPM CHANNEL 1	DPM/100ul - BKG	MEAN DPM/100ul (SD)	TOTAL DPM IN FLUSH	% OF APPLIED DPH	AVG % OF APPLIED DPM (SD)
151	22- 1	Run#1 0.05ppm Flush 1	5223.15	5133.41				
152	22- 2	Run#1 0.05ppm Flush 2	5118.11	5028.36				
153	22- 3	Run#1 0.05ppm Flush 3	5144.08	5054.33	5072.03 54.71	101440.66	97.05	
154	22- 4	Run#2 0.05ppm Flush 1	5003.06	4913.31				
155	22- 5	Run#2 0.05ppm Flush 2	4994.61	4904.86				
156	22- 6	Run#2 0.05ppm Flush 3	5342.80	5253.05	5023.74 198.63	100474.83	96.13	
157	22- 7	Run#3 0.05ppm Flush 1	5321.48	5231.73				
158	22- 8	Run#3 0.05ppm Flush 2	5412.22	5322.47				
159	22- 9	Run#3 0.05ppm Flush 3	5292.32	5202.58	5252.26 62.53	105045.19	100.50	
160	22-10	Run#4 0.05ppm Flush 1	5295.19	5205.44				
161	22-11	Run#4 0.05ppm Flush 2	5358.79	5269.05				
162	22-12	Run#4 0.05ppm Flush 3	5358.01	5268.26	5247.58 36.50	104951.67	100.41	
163	22-13	Run#5 0.05ppm Flush 1	4633.24	4543.49				
164	22-14	Run#5 0.05ppm Flush 2	5609.58	5519.83				
165	22-15	Run#5 0.05ppm Flush 3	5562.56	5472.82	5178.71 550.62	103574.23	99.09	
								98.63 1.98

```

USER#: 0 6                                DATE: FEB 17 1989      Study Number..... MGR8901
ID: MGR-TLC                             TIME: 07:27           Proofed by..... JM 3/8/89
BSF version: 2                          DATE: FEB 22 1989      Lab notebook # and pages... Pages 14-16
Instrument type: LS5801                  TIME: 08:24
Filename:  A:\14C-MGR\WATEXGP2.WK1
File information: 14C-Malachite Green Extraction From Water--Group 2
Extracted from A:\UN06-003.BSF

```

32

14C Malachite Green Extraction From Water - GROUP 2 EXTRACTION-- ANALYSIS OF 0.01 ppm FLUSH SAMPLES

USER#: 0 6 **DATE:** FEB 17 1989 **Study N&r.....** MGR8901
ID: MGR-TLC **TIME:** 07:27 **Proofed by.....** JM 3/8/89
BSF version: 2 **DATE:** FEB 22 1989 **Lab notebook # and pages...** Pages 14-16
Instrument type: LS5801 **TIME:** 08:24
Filename: A:\14C-MGR\WATEXGP2.WK1
File information: 14C-Malachite Green Extraction From Water--Group 2
Extracted from A:\UN06-003.BSF

SAMPLE NUMBER	POSITION NUMBER	SAMPLE ID	DPH CHANNEL 1	DPH/100ul - BKG	MEAN DPM/100ul (SD)	TOTAL DPH IN FLUSH	% OF APPLIED DPH	AVG % OF APPLIED DPH (SD)
166	22-16	Run#1 0.01ppm Flush 1	939.21	849.46				
167	22-17	Run#1 0.01ppm Flush 2	896.31	806.56				
168	22-18	Run#1 0.01ppm Flush 3	995.45	905.70	853.91 49.72	17078.13	80.23	
169	52- 1	Run#2 0.01ppm Flush 1	1073.35	983.60				
170	52- 2	Run#2 0.01ppm Flush 2	1141.70	1051.95				
171	52- 3	Run#2 0.01ppm Flush 3	1163.91	1074.16	1036.57 47.20	20731.45	97.40	
172	52- 4	Run#3 0.01ppm Flush 1	1080.20	990.45				
173	52- 5	Run#3 0.01ppm Flush 2	1159.77	1070.02				
174	52- 6	Run#3 0.01ppm Flush 3	1125.73	1035.98	1032.15 39.93	20643.05	96.98	
175	52- 7	Run#4 0.01ppm Flush 1	764.54	674.80				
176	52- 8	Run#4 0.01ppm Flush 2	572.87	483.13				
177	52- 9	Run#4 0.01ppm Flush 3	1262.28	1172.53	776.82 355.84	15536.35	72.99	
178	52-10	Run#5 0.01ppm Flush 1	1055.96	966.22				
179	52-11	Run#5 0.01ppm Flush 2	1178.99	1089.25				
180	52-12	Run#5 0.01ppm Flush 3	1048.75	959.00	1004.82 73.20	20096.45	94.41	
								88.40
								11.12

```

USER#: 06                                DATE: FEB 17 19                Study Number..... MGR8901
ID: HGR-TLC                            TIME: 07:27                   Proofed by..... JM 3/8/89
BSF version: 2                          DATE: FEB 22 19                Lab notebook # and pages... Pages 14-16
Instrument type: LS5801                  TIME: 08:24
Filename:  A:\14C-MGR\WATEXGP2.WK1
File information:  14C-Malachite Green Extraction Fran Water--Group 2
Extracted from A:\UN06-003.BSF

```

34

14C Malachite Green Extraction From Water - GROUP 2 EXTRACTION-- ANALYSIS OF 0.005 ppm FLUSH SAMPLES

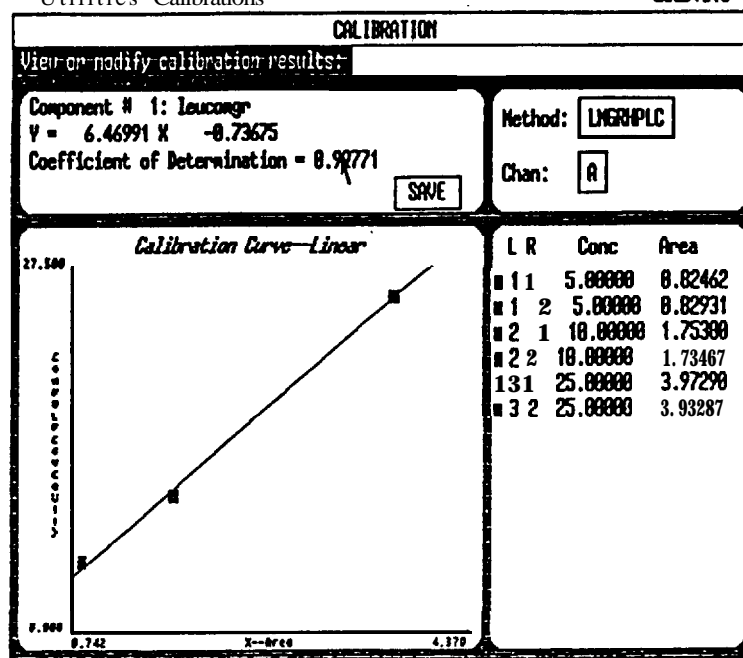
USER#: 06 DATE: FEB 17 1989 Study Number..... MGR8901
 ID: MGR-TLC TIME: 07:27 Proofed by..... JM 3/8/89
 BSF version: 2 DATE: FEB 22 1989 Lab notebook # and pages... Pages 14-16
 Instrument type: LS5801 TIME: 08:24
 Filename: A:\14C-MGR\WATEXGP2.WK1
 File information: 14C-Malachite Green Extraction From Water--Group 2
 Extracted from A:\UN06-003.BSF

SAMPLE NUMBER	POSITION NUMBER	SAMPLE ID	DPR CHANNEL 1	DPM/100ul - BKC	MEAN DPM/100ul (SD)	TOTAL DPM IN FLUSH	% OF APPLIE DPM	AVG % OF APPLIED DPM (SD)
181	52-13	Run#1 0.005ppm Flush 1	508.10	418.35				
182	52-14	Run#1 0.005ppm Flush 2	549.95	460.20				
183	52-15	Run#1 0.005ppm Flush 3	555.72	465.98	448.18 25.99	8963.51	98.51	
184	52-16	Run#2 0.005ppm Flush 1	593.27	503.52				
185	52-17	Run#2 0.005ppm Flush 2	580.82	491.07				
186	52-18	Run#2 0.005ppm Flush 3	586.45	496.71	497.10 6.23	9941.98	109.27	
187	12- 1	Run#3 0.005ppm Flush 1	578.23	488.48				
188	12- 2	Run#3 0.005ppm Flush 2	558.67	468.92				
189	12- 3	Run#3 0.005ppm Flush 3	591.36	501.62	486.34 16.45	9726.80	106.90	
190	12- 4	Run#4 0.005ppm Flush 1	583.49	493.74				
191	12- 5	Run#4 0.005ppm Flush 2	605.58	515.84				
192	12- 6	Run#4 0.005ppm Flush 3	611.87	522.12	510.57 14.91	10211.36	112.23	
193	12- 7	Run#5 0.005ppm Flush 1	601.03	511.29				
194	12- 8	Run#5 0.005ppm Flush 2	596.22	506.47				
1 %	12- 9	Run#5 0.005ppm Flush 3	589.34	499.60	505.78 5.87	10115.69	111.18	
								107.62 5.48

Appendix C
Analytical Methods for Malachite Green
Project 89-040

Standard curves and chromatograms of malachite green and leuco malachite green from well water samples spiked with 10 ppb, description of the calculation of ppb from ng found by HPLC, and the recovery of malachite green residues from well water samples spiked with 10 ppb each of malachite green and leuco malachite green.

(A).



(B).

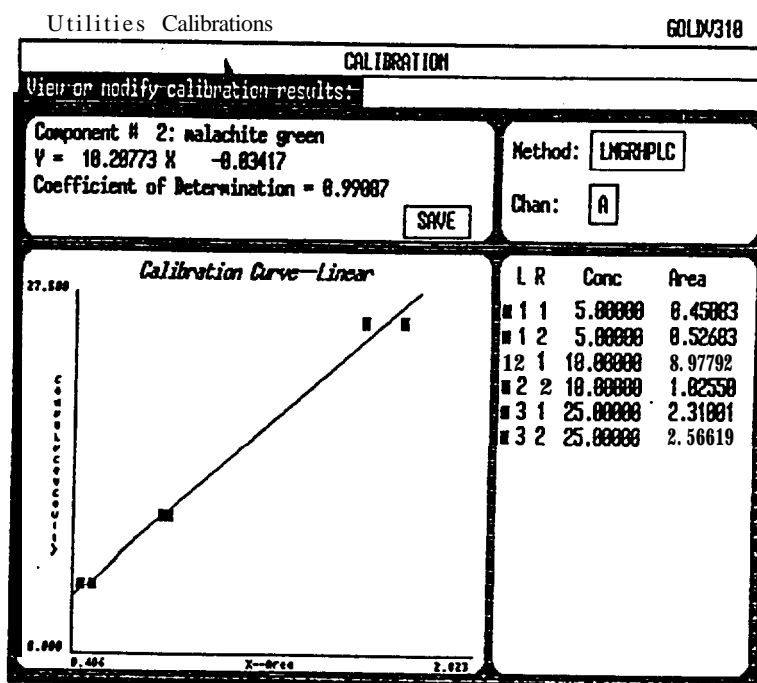


Figure C.1. HPLC standard curves for leuco malachite green (A) and malachite green (B).

COLLECTION DATA LHMSTB10 A 2 2 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LHMPLC C:\GOLD\SYSTEM\METHOD\
 REPORT DATA LHMSTB10 A 2 2 Orig C:\GOLD\SYSTEM\DATA\10OCT90\
 METHOD LHMPLC C:\GOLD\SYSTEM\METHOD\

TIME DATE
 INJECTION 09:39:36 10 OCT 1990
 REPORT 10:44:21 30 APR 1991

SYSTEM 1

Analyst John Allen, Jane Gofus, and Mary Thompson
 Comments Study NGR901
 MALACHITE GREEN AND LEUCO MALACHITE am 10 NG STANDARD
 PAGE 40
 DISK: NGR901-04 FILE: A:\DIOLETR\10OCT90\LHMSTB.SMP
 File Folder: NGR901-34

Type	Sample Name	Sample Amount	Int Std Amount	Scale Factor	Mr. Inj	Vial Mr.	Inject Vol uL	
Cal	LHMSTB10			1.00000	2 / 2		50	(from File)
Peak Number	Retention Time	Component Name	Concentration ng	Peak Area	Peak Height	Response Factor	Area Percent	Height Percent
1	4.503	leucoagr	10.0000	1.73467	0.00566	CAL	62.846	86.129
2	12.279	malachite green	10.0000	1.02350	0.00091	CAL	37.154	13.871
TOTALS			20.0000	2.76018	0.00657		100.000	100.000

TIME DATE
 INJECTION 09:39:36 10 OCT 1990
 REPORT 10:47:39 30 APR 1991

COLLECTION DATA LHMSTB10 A 2 2 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LHMPLC C:\GOLD\SYSTEM\METHOD\
 REPORT DATA LHMSTB10 A 2 2 Orig C:\GOLD\SYSTEM\DATA\10OCT90\
 METHOD LHMPLC C:\GOLD\SYSTEM\METHOD\

SYSTEM 1

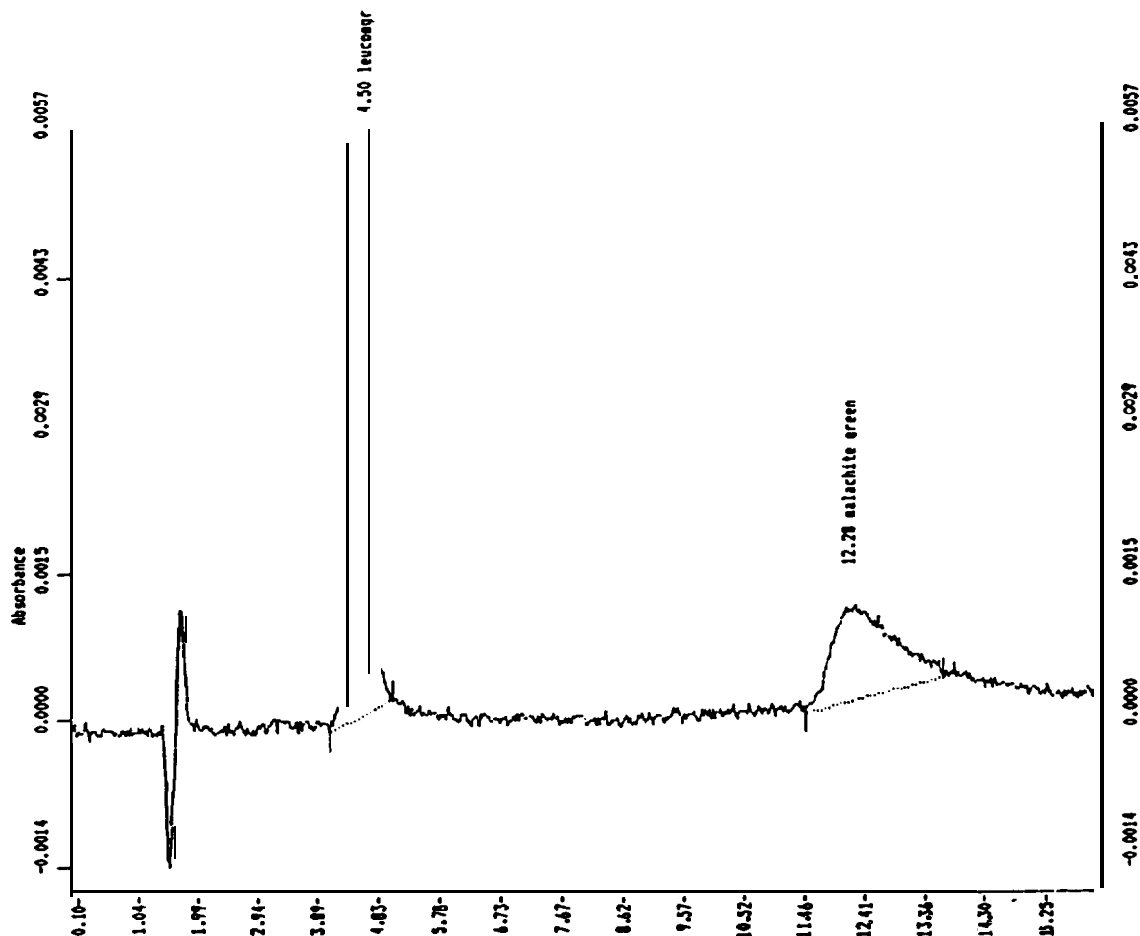


Figure C.2. HPLC analysis and chromatogram for a leuco malachite green and malachite green 10-ng standard made up in mobile phase.

COLLECTION	DATA	NAME	CHAM	LEV	REP	TYPE	DIRECTORY	TIME	DATE
		HINDET10	A	1	2	Orig	C:\GOLD\SYSTEM\DATA\	INJECTION	12:37:34 10 OCT 1990
	METHOD	LINSMPLC					C:\GOLD\SYSTEM\METH\		
REPORT	DATA	HINDET10	A	1	2	Orig	C:\GOLD\SYSTEM\DATA\10OCT90\	REPORT	14:27:11 26 APR 1991
	METHOD	LINSMPLC					C:\GOLD\SYSTEM\METH\		

SYSTEM 1

Analyst John L. Allen and Jane E. Gofus

Comments Study NGR8901

BIOLOGICAL EXTRACTION OF MALACHITE GREEN RESIDUES FROM TAP WATER

PAGE 60

DISK: NGR8901-04 FILE : A:\BIOLETR\10OCT90\HINDET10.SMP

File Folder: NGR8901-34

Type	Sample Name	Sample Amount	Int Std Amount	Scale Factor	Nr. Inj	Vial Nr.	Inject Vol uL	
Unit	HINDET10			1.00000	2 / 2		50	(from File)

Peak Number	Retention Time	Component Name	Concentration ng	Normalized Concentration	Peak Area	Peak Height	Base Code	Response Factor	Rel. Ret Time	Ret Area Percent	Height Percent
1	4.439	leucoagr	5.5401	27.419	0.97017	0.00312	u o	6.4699	0.0000	40.252	73.710
2	11.438	malachite green	14.6655	72.581	1.44006	0.00111	BCB	10.2077	0.0000	59.748	26.290
TOTALS			20.2057	100.000	2.41022	0.00424				100.000	100.000

TIME DATE

INJECTION 12:37:34 10 OCT 1990

REPORT 14:27:11 26 APR 1991

NAME CHAM LEV REP TYPE DIRECTORY

COLLECTION DATA HINDET10 A 1 2 Orig C:\GOLD\SYSTEM\DATA\

METHOD LINSMPLC C:\GOLD\SYSTEM\METH\

REPORT DATA HINDET10 A 1 2 Orig C:\GOLD\SYSTEM\DATA\10OCT90\

METHOD LINSMPLC C:\GOLD\SYSTEM\METH\

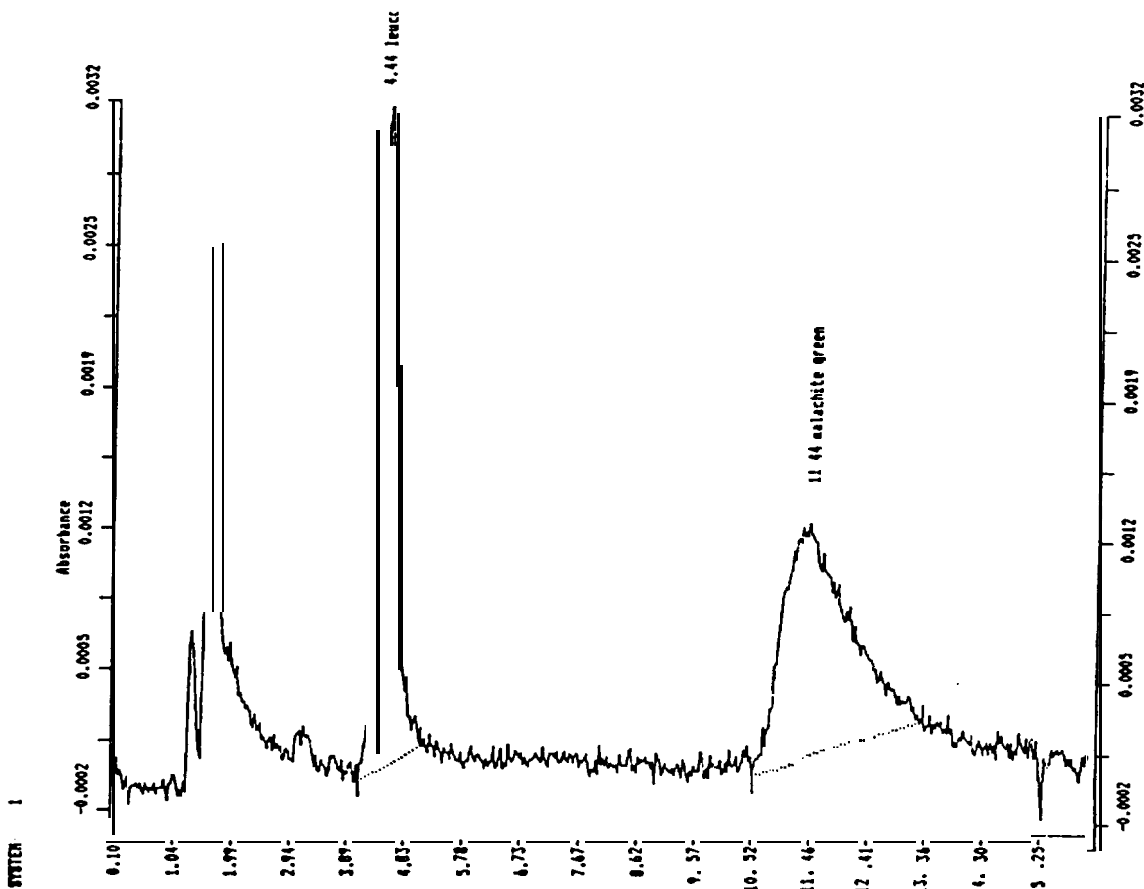


Figure C.3. HPLC analysis and chromatogram of a 10-ppb sample extract.

Table C.1. Example of the calculation to convert HPLC nanograms to parts per billion.

The 50-mL water samples were concentrated on the Baker 10 Diol column and eluted from the column with 2.0 mL of 0.05 M p-toluene sulfonic acid in methanol. A 0.05-mL HPLC injection was made. The concentrations of leuco malachite green and malachite green were calculated from the ng found by HPLC using the following formula:

$$\text{ppb of leuco malachite green} = \frac{\text{ng leuco malachite green}}{(50 \text{ mL}/2.0 \text{ mL})(0.05 \text{ mL})}$$

$$\text{ppb of malachite green} = \frac{\text{ng malachite green}}{(50 \text{ mL}/2.0 \text{ mL})(0.05 \text{ mL})}$$

Table C. 2. Recovery of malachite green residues from well water samples spiked with 10 ppb each of malachite green (MGR) and leuco malachite green (LEUCO).

Sample number	Leuco		MGR		Leuco t MGR	
	Conc. recovered (ppb)	Percent recovered	Conc. recovered (ppb)	Percent recovered	Conc. recovered (ppb)	Percent recovered
1	5.38	53.8	12.1	121	17.5	87.5
2	4.55	45.5	10.9	109	15.5	77.3
3	4.39	43.9	10.7	107	15.1	75.3
4	4.30	43.0	11.7	117	16.0	80.2
5	4.01	40.1	10.9	109	14.9	74.6
6	4.49	44.9	9.90	98.7	14.4	71.8
7	4.38	43.8	10.5	105	14.9	74.3

Appendix D
Analytical Methods for Malachite Green
Project 89-040

<p>HPLC Minimum Detection Limit Raw Data for Chromatic and Leuco Malachite Green</p>
--

Table D.1. Raw data from the method detection limit (MDL) determination for the HPLC analysis of leuco malachite green (LEUCO) and malachite green (MGR) with diol-column enrichment according to the EPA procedure for the determination of the method detection limit.

Sample number	Leuco		MGR		Leuco + MGR	
	Conc. recovered (ppb)	Percent recovered	Conc. recovered (ppb)	percent recovered	Conc. recovered (ppb)	Percent recovered
1	12.1	48.5	20.0	80.1	32.2	64.3
2	13.2	52.6	20.6	82.4	33.8	67.5
3	11.2	44.8	18.3	73.0	29.4	58.9
4	11.8	47.4	18.6	74.5	30.5	60.9
5	11.7	46.8	19.2	76.6	30.9	61.7
6	12.6	50.4	20.2	80.6	32.8	65.5
7	11.8	47.2	20.3	81.0	32.1	64.1

Appendix E
Analytical Methods for Malachite Green
Project 89-040

**Chromatograms of Well Water Samples Spiked
with the Carbinol and Leuco Forms of
Malachite Green**

COLLECTION DATA CARB2 A 1 2 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LINGRPLC C:\GOLD\SYSTEM\DATA\
 REPORT DATA CARB2 A 1 2 Orig C:\GOLD\SYSTEM\DATA\11OCT90\
 METHOD LINGRPLC C:\GOLD\SYSTEM\DATA\

TIME DATE
 INJECTION 11:28:02 11 OCT 1990
 REPORT 14:09:37 26 APR 1991

SYSTEM 1

Analyst John L. Allen and Jane E. Bufus
 Comments Study NGR8901
 BIOD. EXTRACTION OF CARBINOL FROM TAP WATER
 PAGE 60-61
 DISK: NGR8901-04 FILE: A:\BIOLETR\11OCT90\CARB2.SMP
 File Folder: NGR8901-37

Type	Sample Name	Sample Amount	Int Std Amount	Scale Factor	Nr. Inj	Vial Nr.	Inject Vol uL				
Unt	CARB2			1.00000	2 / 2		50	(from File)			
Peak Number	Retention Time	Component Name	Concentration ng	Normalized Concentration	Peak Area	Peak Height	Base Code	Response Factor	Rel. Ret Time	Ret Area Percent	Height Percent
=====											
	4.681	leucogsr									
1	9.9430	alachitgreen	74.1903	100.000	9.62237	0.00869	BCB	6.8598	0.0000	100.000	100.000
=====											
TOTALS			74.1903	100.000	9.62237	0.00869				100.000	100.000

NAME CHAN LEV REP TYPE DIRECTORY
 COLLECTION DATA CARB2 A 1 2 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LINGRPLC C:\GOLD\SYSTEM\DATA\
 REPORT DATA CARB2 A 1 2 Orig C:\GOLD\SYSTEM\DATA\11OCT90\
 METHOD LINGRPLC C:\GOLD\SYSTEM\DATA\
 TIME DATE
 INJECTION 11:28:02 11 OCT 1990
 REPORT 14:09:37 26 APR 1991

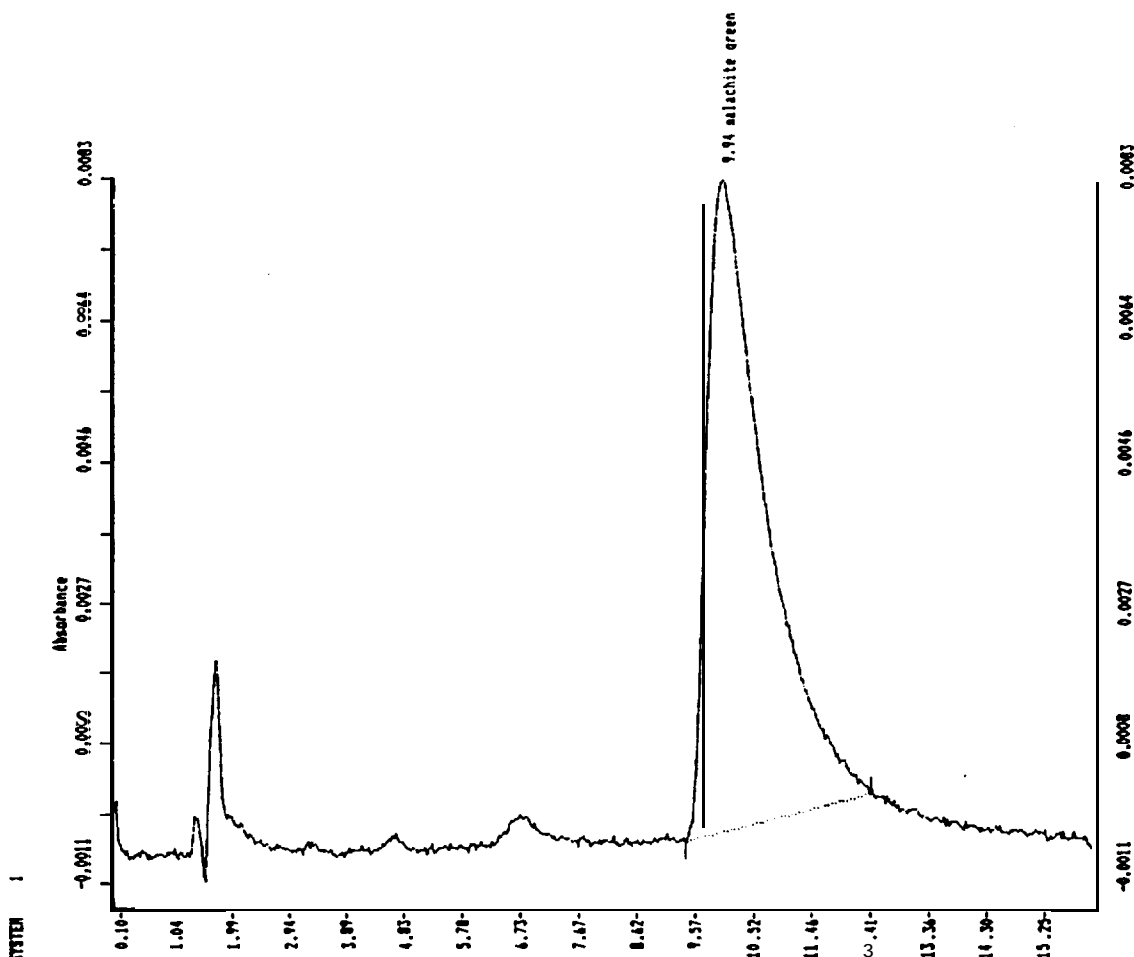


Figure E.1. HPLC chromatogram and analysis of a well water sample spiked with 50-ppb malachite green carbinol.

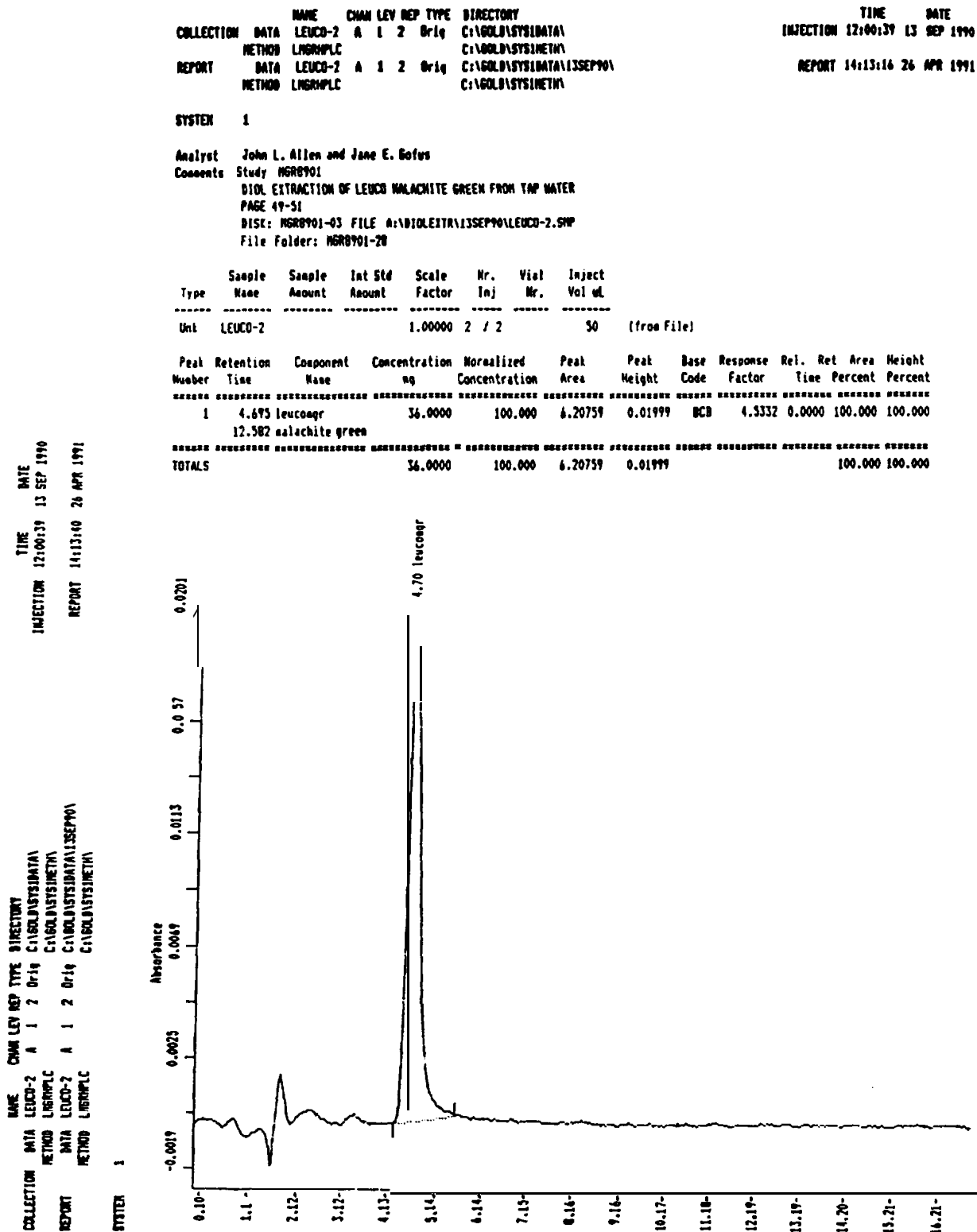


Figure E.2. HPLC chromatogram and analysis of a well water sample spiked with 50-ppb leuco malachite green.

Appendix F
Analytical Methods for Malachite Green
Project 89-040

<p>Recovery of Malachite Green Residues from Water of Various pHs</p>
--

Table F.1. Recoveries of malachite green residues from deionized water samples buffered to pH 6.0, 7.0, and 9.0 and well water samples with a pH of 8.0. All samples were spiked with 50.0 ppb of leuco malachite green (LEUCO) and 50.0 ppb of malachite green (MR).

pH	Sample number	Leuco		MR		Leuco + MR	
		Conc. recovered (ppb)	Percent recovered	Conc. recovered (ppb)	Percent recovered	Conc. recovered (ppb)	Percent recovered
6	1	31.4	62.8	47.8	95.5	79.2	79.2
	2	31.4	62.9	50.6	101	82.1	82.1
	3	28.9	57.9	49.1	98.1	78.0	78.0
7	1	26.4	52.8	47.4	94.8	73.8	73.8
	2	25.1	50.2	47.5	95.1	72.6	72.6
	3	23.7	47.3	42.6	85.2	66.2	66.2
8	1	28.1	56.3	50.9	102	79.0	79.0
	2	26.7	53.3	48.9	97.9	75.6	75.6
	3	27.2	54.5	53.5	107	80.7	80.7
	4	23.5	47.1	49.1	98.2	72.7	72.7
	5	20.9	41.7	46.8	93.7	67.7	67.7
	6	18.3	36.6	45.7	91.4	64.0	64.0
9	1	19.9	39.8	48.8	97.7	68.8	68.8
	2	19.0	38.1	50.8	102	69.8	69.8
	3	19.6	39.2	49.4	98.7	69.0	69.0

Appendix G
Analytical Methods for Malachite Green
Project 89-040

**Chromatogram of malachite green residues from pond water
analyzed by HPLC with diol-column enrichment, recovery of malachite green
residues from pond water samples spiked at 25.0, 50.0, and 100 ppb each of
leuco malachite green and malachite green, and the water chemistry
analysis of NFLX pond water**

NAME CHAN LEV REP TYPE DIRECTORY
 COLLECTION DATA POND-2 A 1 1 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LNRHPLC C:\GOLD\SYSTEM\DATA\
 REPORT DATA POND-2 A 1 1 Orig C:\GOLD\SYSTEM\DATA\19SEP90\
 METHOD LNRHPLC C:\GOLD\SYSTEM\DATA\

TIME DATE
 INJECTION 12:48:11 19 a 1990
 REPORT 11:11:33 30 APR 1991

SYSTEM 1

Analyst John L. Allen and Jane E. Gofus
 Comments Study NGR8901
 DIOL EXTRACTION OF MALACHITE GREEN RESIDUES FROM POND WATER
 PAGE 32
 DISK: NGR8901-03 FILE : A:\DIOL\EXTR\19SEP90.SMP
 File Folder: NGR8901-29

Type	Sample Name	Sample Amount	Int Amount	Std Factor	Scale	Nr. Inj	Vial Nr.	Inject Vol ul
------	-------------	---------------	------------	------------	-------	---------	----------	---------------

Unk	Pow-z			1.0000	1 / 2	50	(from File)	
-----	-------	--	--	--------	-------	----	-------------	--

Peak Number	Retention Time	Component Name	Concentration ng	Normalized Concentration	Peak Area	Peak Weight	Base Code	Response Factor	Rel. Time	Ret Area	Height Percent
1	5.759	leucoagr	77.4259	37.911	11.06329	0.02948	BCD	4.2856	0.0000	33.211	59.546
2	10.919	malachitegreen	126.0023	62.089	22.28919	0.02001	BCD	5.4266	0.0000	66.789	40.454
TOTALS			204.2282	100.000	13.37248	0.04959				100.000	100.000

TIME DATE
 INJECTION 12:48:11 19 SEP 1990
 REPORT 11:12:03 30 APR 1991

NAME CHAN LEV REP TYPE DIRECTORY
 COLLECTION DATA POND-2 A 1 1 Orig C:\GOLD\SYSTEM\DATA\
 METHOD LNRHPLC C:\GOLD\SYSTEM\DATA\
 REPORT DATA POND-2 A 1 1 Orig C:\GOLD\SYSTEM\DATA\19SEP90\
 METHOD LNRHPLC C:\GOLD\SYSTEM\DATA\

SYSTEM 1

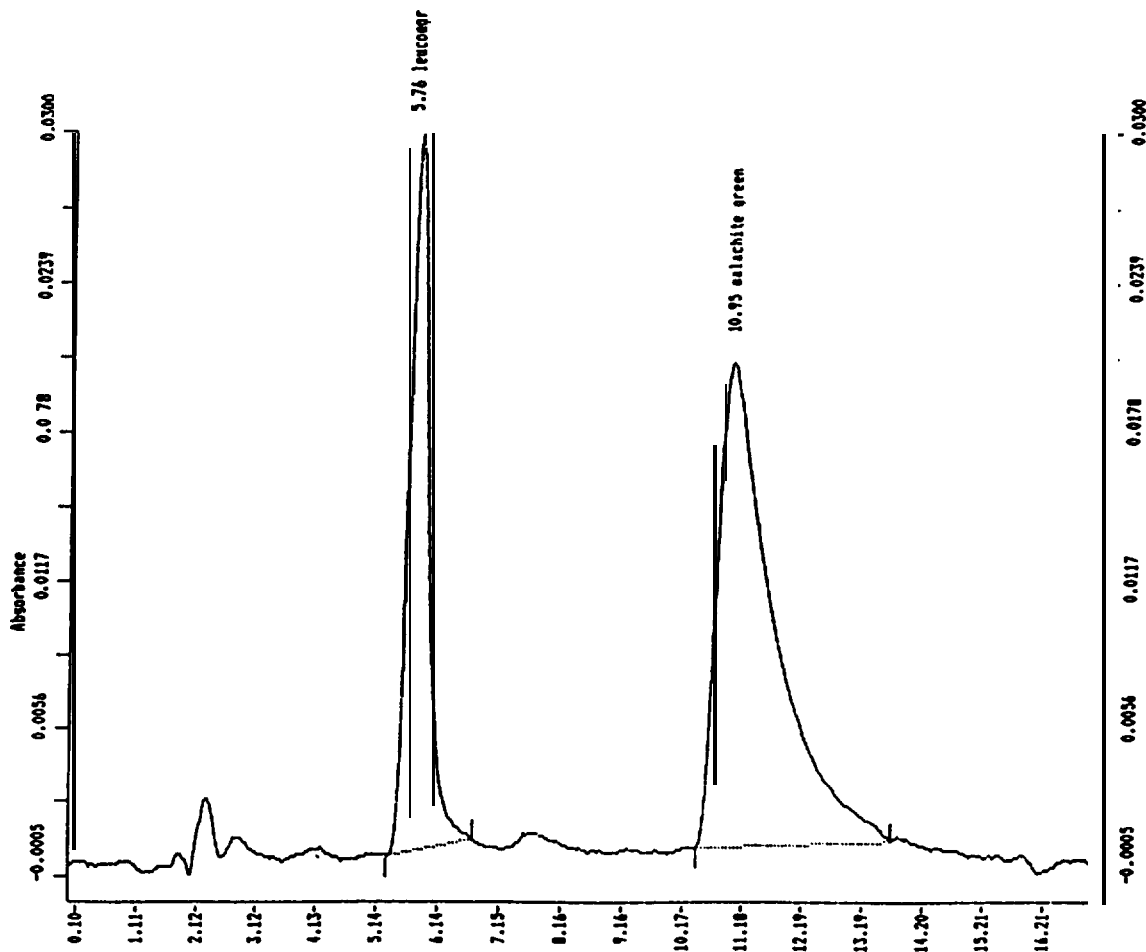


Figure G.1. HPLC chromatogram and analysis of pond water sample spiked with 100-ppb leuco malachite green and malachite green.

Table G.1. Recovery of malachite green residues from pond water spiked at concentrations of 25.0, 50.0, and 100 ppb each of leuco malachite green (LEUCO) and malachite green (MGR).

Sample number	Spike conc. (ppb)	Leuco		MGR		Leuco t MGR	
		Conc. Recovered (ppb)	Percent recovered	Conc. recovered (ppb)	percent recovered	Conc. recovered (ppb)	Percent recovered
1	25.0	14.9	59.6	24.8	99.0	39.7	79.3
2	25.0	14.4	57.4	23.3	93.4	37.7	75.4
3	50.0	29.7	59.4	46.7	93.5	76.4	76.4
4	50.0	27.9	55.8	48.1	96.2	76.0	76.0
5	50.0	25.1	50.1	44.6	89.2	69.7	69.7
6	100	63.1	63.1	94.5	94.5	158	78.8
7	100	61.5	61.5	98.9	98.9	160	80.2
8	100	62.9	62.9	97.6	97.6	161	80.3

Table 6.2. Water chemistry analysis of NFLX pond water.

**Water Chemistry Data
for John Allen**

Sample	pH	Alkalinity	Hardness	Ammonia	Nitrate*	Nitrite
3	7.75	110	138	0.10	3.68	0.01
3			138		4.49	0.02
4	7.77	111	138	0.10	3.29	0.01
4	7.89	112		0.09		

* The auto dilution loop on the auto-analyzer gave bumpy peaks for Nitrates above 2 mg N/L. Nitrate data is only approximate.